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Importance of oxidatively modified proteins in chronic renal failure

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Importance of oxidatively modified proteins in chronic renal failure. Considerable evidence has accumulated that chronic uremia is associated with a multifactorial immunoinflammatory syndrome, which occurs early in the course of renal failure, is accentuated with the progression of uremia, and culminates in maintenance dialysis therapy. We previously described the presence of a circulating oxidized plasma protein named advanced oxidation protein products (AOPPs). Beyond evidence that AOPPs represent an exquisite marker of oxidative stress, their role(s) in the pathophysiology of chronic renal failure and dialysis-related complications might be of great importance. Regarding the mechanisms of generation of AOPP, we underscore the importance of the chlorinated oxidants, previously solely considered as microbicidal agents, in the generation of AOPP. Indeed, AOPPs appear to act as true inflammatory mediators since they are able to trigger the oxidative burst in neutrophils as well as in monocytes. Thus, it is hypothesized that the AOPPs, which arise from the reaction between chlorinated oxidants and plasma proteins, constitute a new molecular basis for the deleterious activity of oxidants, and they could be considered to be true mediators of the proinflammatory effect of oxidative stress in uremia.

Oxidative stress has long been incriminated in the development of the dialysis-related pathology [1], in particular β_2-microglobulin amyloid arthropathy, and accelerated atherosclerosis responsible to a considerable degree for the morbidity and mortality in the dialysis population. In our recent work, the aim of which was to characterize the pathophysiology of dialysis-related oxidative stress better, we first described the presence of oxidatively modified proteins in the plasma of dialysis patients. We designated these compounds advanced oxidation protein products (AOPP), by analogy with the well-characterized advanced glycation end products (AGEs) with which AOPP appeared to be closely related structurally [2]. We then showed that high circulating levels of AOPP in fact are also present in the plasma of nondialyzed chronic renal failure (CRF) patients and reflect the degree of monocyte activation [3]. Taken together, these findings led us to propose AOPPs not only as novel markers of oxidative stress, but also as potential mediators of inflammation in CRF [4].

The present brief review successively considers (*1*) the protagonists and molecular basis of oxidative stress and the evidence of such a stress in CRF patients, whether or not they are on maintenance dialysis; (*2*) the susceptibility of proteins to oxidant-mediated damage and the biochemical characteristics of AOPP; and (*3*) the potential role of AOPPs in CRF-associated immunoinflammatory disorders.

PROTAGONISTS AND MOLECULAR BASIS OF OXIDATIVE STRESS

Oxidative stress is defined as a disruption of the equilibrium between the generation of oxidants and the activity of antioxidant systems. Such a definition also needs to incorporate its damage products toward elementary vital structures, such as lipids, DNA, and proteins [5]. Oxidants can be generated in increased amounts by a number of mechanisms at multiple cellular sites. In the present context of CRF and dialysis, the major source of oxidants is afforded by circulating polymorphonuclear (PMN) neutrophils and monocytes, when activated by uremic toxins or bioincompatible dialysis membranes.

Phagocyte-derived oxidants

It has long been known that following appropriate stimulation, both polymorphonuclear (PMN) neutrophils and monocytes develop a respiratory burst that leads to a massive generation of highly reactive oxygen species (ROS), which play a key roll in the host defense against pathogens and tumoral cells. Excessive ROS also may damage normal structures [6, 7]. At the molecular level, the reaction is generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is dormant in the resting phagocyte. Following activation, however, NADPH assembles its distinct molecular components in an electron transfer chain capa-

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ble of performing a univalent reduction of molecular oxygen in superoxide anion ($O_2^{\cdot-}$) at the expense of itself [8]. Superoxide anion is almost immediately converted [spontaneously or by superoxide dismutase (SOD)] into hydrogen peroxide (H_2O_2), which produces single oxygen and hydroxyl radicals ($OH^{\cdot}$) in the presence of iron. Superoxide anion and hydrogen peroxide are themselves not particularly potent at killing microorganisms, but are used by phagocytes as precursors for the production of more powerful oxidants [9]. For example, superoxide anion interacts with nitric oxide (NO) to form highly toxic nitrogen derivatives (peroxynitrite) [10], while H_2O_2, which is able to cross plasma membranes, reacts with intracellular iron to form hydroxyl radicals by a series of interactions termed the Haber-Weiss cycle. Hydroxyl radicals have a large spectrum of toxicity, affecting most cellular components. In particular, they trigger peroxidation of cell membrane lipids, promote protein aggregation, and cause damage leading to mutation and/or cleavage of DNA [11–13]. In addition to the formation of ROS, phagocytic cells retain the unique capacity to produce chlorinated oxidants caused by myeloperoxidase, which in the presence of chloride (Cl^-) converts hydrogen peroxide into hypochlorous acid (HOCl) [9]. Hypochlorous acid is the most toxic and the most reactive species formed by phagocytic cells. Its elective targets remain the membrane proteins and notably their thiol groups. Oxidation of intracellular enzymes of nucleotides and cytochromes by HOCl leads to an inhibition of essential living processes such as the respiratory chain. Finally, HOCl may react with endogenous amines (R-NHI) to generate chloramines (RNH-Cl), also termed "long-lived oxidants" in order to distinguish them from oxygen free radicals whose life span is extremely short [14]. The generation of oxidants by phagocytic cells can be easily measured directly within whole blood by chemiluminescence (CL) [15]. Depending on the CL probe used, one may quantitate either NADPH oxidase activity alone, that is, the production of superoxide anion (as measured by lucigenin-amplified CL), or the intracellular production of hydrogen peroxide and myeloperoxidase-dependent formation of chlorinated oxidants (as measured by luminol-amplified CL).

Physiologic protection against oxidants

Physiologic protection against oxidants involves both enzymatic antioxidant systems, including SOD, catalase and glutathione peroxidase, and antioxidant molecules without enzymatic activities, which are also called scavengers and comprise: glutathione disulfide-containing tripeptide, present in all cell types and capable of scavenging H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$, $^1O_2$, and chlorinated oxidants; α-tocopherol (vitamin E), which is localized mainly in the cell membrane in a strategic position for protecting it from lipid peroxidation; ascorbic acid (vitamin C), widely distributed in both intracellular and extracellular media, directly scavenging $O2_2^{\cdot-}$ and $OH^{\cdot}$ by forming semideshydro-ascorbic acid, itself scavenged by glutathione; and cysteine, taurine, and methionine, which selectively scavenge hypochlorous acid and chloramines. In addition, uric acid, glucose, and mannitol also retain the capacity to neutralize some oxidants; ferritin, transferrin, and ceruloplasmin may also exert antioxidant effects by sequestering transition metal ions, and thus limit the formation of $OH^{\cdot}$ via the Haber-Weiss cycle [16].

OXIDATIVE STRESS IN CRF

Conditions for the generation of oxidative stress are generally present in the CRF patient on maintenance dialysis in whom an intermittent generation of oxidants occurs recurrently and in combination with a chronic antioxidant deficiency.

Increased generation of oxidants

It has long been known that a dialysis session with cellulosic dialysis membranes (for example, cuprophane), but not with synthetic membranes (such as polyacrilonitrile AN69), triggers a massive increase in the phagocyte basal production of ROS [17]. The peak of CL production occurs simultaneously with the nadir of the well-known neutropenia and is closely related to the amount of C5a and C3a generation [18]. Other authors, using flow cytometry, also reported the increased generation of intracellular ROS in both monocytes and PMN cells during dialysis sessions, that was closely related to the membrane biocompatibility [19]. Interestingly, this activation of phagocyte respiratory burst is associated with an increased expression of adhesion molecules, which is closely dependent on the bioincompatibility of the dialysis membranes [20].

Due to the use of more biocompatible dialysis membranes, such a close relationship between production of oxidants and dialysis membrane biocompatibility, widely documented in the literature [21], has become more difficult to assess in the dialysis patient.

Decreased antioxidant potential

Profound deficiencies in the activity of the glutathione system and in selenium have been reported in dialysis patients [22–24]. In our own study, we observed that glutathione peroxidase activity is significantly altered at an early stage of CRF, regularly decreases with the progression of uremia, and is dramatically reduced in the dialysis patient in whom it is associated with a markedly reduced level of glutathione [24, 25]. More conflicting data have been reported regarding the levels and/or the activities of SOD, trace elements (for example, copper and zinc), and other oxidant-scavenging molecules (such as ceruloplasmin), and transferrin, in CRF patients. Vita-

min C deficiency has been observed in CRF patients because of the dietary restriction of fresh fruit and vegetables to avoid hyperkalemia and because of vitamin C loss during dialysis. However, others reported plasma vitamin C concentrations within the normal range in the absence of any supplementation. Whether vitamin C by itself exerts a pro-oxidant [26] or antioxidant effect [27] remains a matter of debate.

In contrast, plasma vitamin E concentrations in CRF patients have been shown to be usually normal, whereas erythrocyte and mononuclear cell concentrations appear to be decreased [28]. Nevertheless, vitamin E oral supplementation or dialysis with vitamin E-modified membranes was shown to protect against oxidative stress during hemodialysis [29, 30].

Oxidative stress markers

Until recently, direct evidence for in vivo oxidative stress in the dialysis patient almost solely relied on the measurement of lipid peroxidation by-products such as malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS; refer to the companion article by Drüeke et al in this Supplement of *Kidney International*, p. S-114) [31–34].

Surprisingly and despite the exquisite susceptibility of proteins to oxidants, the search for the presence of oxidatively modified proteins in the plasma of hemodialyzed patients had not been extensively developed until our recent description of this novel protein oxidation marker, referred to as AOPPs, in the plasma of these patients.

OXIDATIVELY MODIFIED PROTEINS

Susceptibility of proteins to oxidants

It has been established that proteins represent elective targets of oxidant-mediated injury [12, 35]. Biochemical and structural modifications of proteins induced by oxidative attack may lead to functional alterations and in particular to the progressive loss of their metabolic, enzymatic, or immunologic properties. In vitro exposure of proteins to oxidants induces alterations in their primary, secondary, or tertiary structure, which vary depending on the type of oxidant. For a given oxidant, depending on the intensity of the oxidative attack, these modifications may go from the oxidation of a single amino acid residue, to the fragmentation or complete denaturation of the protein across intermediary steps of increased hydrophobicity, augmented susceptibility, or resistance to proteolysis.

In fact, whereas moderate oxidation of proteins leads to augmentation of hydrophobicity and favors their catabolism by the multicatalytic or proteasome complex, intense oxidation generates insoluble products that resist to proteolysis [36, 37]. Generation of dityrosine, which results from covalent binding links between two tyrosine residues, is a selective marker of protein attack [37–39]. Formation of carbonyls represents another early marker of protein oxidation. It involves cations of the redox cycle such as Fe^{++} and Cu^{++}, which have binding sites on proteins and may transform amino acid residues in carbonyls in the presence of hydrogen peroxide and superoxide anion, the amino acids lysine, arginine, proline, and histidine being the most prone to generate carbonyls.

Our previous study, which analyzed the structural modifications of β_2-microglobulin induced by ROS generated by water pulse radiolysis, clearly illustrated this selectivity of the protein damage depending on the type of oxidant [40]. While hydroxyl radical alone induces aggregation and conformational changes of β_2-microglobulin, the combination of hydroxyl radical with equimolar concentrations of superoxide anion induces its fragmentation. The loss of tryptophan and the production of dityrosine follow these structural modifications induced by ROS in a dose-dependent manner.

Advanced oxidation protein products

Biochemical characteristics. The strategy that led us to identify AOPP in the plasma of dialysis patients consisted of fractionating this plasma by exclusion chromatography and searching for spectral modifications in the resulting fractions [2]. This procedure led to an identification of two absorption peaks at 340 nm, corresponding to molecular mass of 600 kD [high molecular weight (HMW-AOPP)] and 80 kD [low molecular weight (LMW-AOPP)], while no such peaks were found in normal subjects. Protein electrophoresis showed that the HMW-AOPP peak is mostly due to albumin, which appears to form aggregates likely resulting from disulfide bridges and/or dityrosine cross-linking. Likewise, the LMW-AOPP peak also contains albumin in its monomeric form. To determine the spectral characteristics of AOPP, ultraviolet absorbance and fluorescence emission spectra of native human serum albumin (HSA) and HSA treated with chlorinated oxidants (HSA-AOPP) were analyzed and compared with those of purified dityrosine. This led us to conclude that dityrosine effectively represents the main chromophore of AOPP with an absorption at 315 nm and an emission band at 410 nm (after excitation at 315 nm).

AOPP: Elective markers of oxidative stress. Our study of a large series of dialysis patients showed that as compared with healthy subjects, all had increased plasma concentrations of AOPP. Interestingly, in predialysis patients with severe CRF, plasma levels of AOPP were also significantly increased, although lower than in hemodialyzed patients [2], suggesting that uremia per se may induce a state of oxidative stress. This was verified in a more recent study of a large cohort of nondialyzed uremic patients at various stages of CRF [3]. Indeed, we found that AOPP plasma levels were already increased

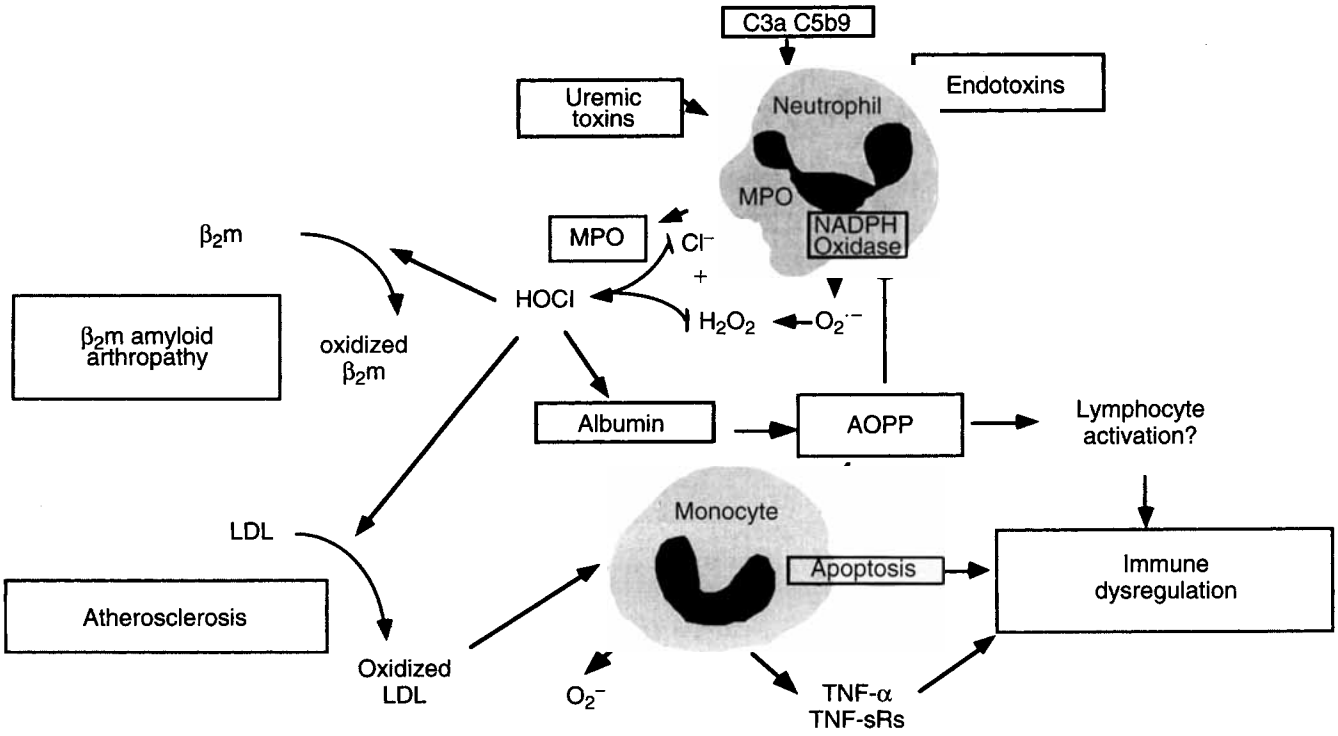

Fig. 1. Pathophysiological role of advanced oxidized protein products (AOPP). Abbreviations are: β_2m, β_2-microglobulin; MPO, myeloperoxidase; LDL, low density lipoprotein; TNF-α, tumor necrosis factor-α; NADPH, nicotinamide adenine dinucleotide phosphate.

at a mild stage of CRF and gradually increased with the progression of renal failure, as exemplified by a strong negative correlation between the plasma levels and creatinine clearance. Plasma levels of MDA were also higher in CRF patients than in healthy controls, but remained stable regardless of the degree of renal deterioration. These findings strongly supported the dual concept that oxidative stress exists long before maintenance dialysis and that AOPPs are more accurate oxidative stress markers than oxidized lipid products.

AOPP: Mediators of inflammation. As already mentioned earlier in this article, a close correlation was found in both dialyzed and nondialyzed CRF patients between AOPP and the AGE pentosidine [2, 3]. AGEs exert numerous biological activities, in particular proinflammatory actions, and have been proposed as a novel class of uremic toxins as their levels closely reflect the progression of CRF [39]. The implication of increased oxidative stress in the formation of AGE and the hypothesis that protein oxidation might contribute to the effects attributed so far to AGE have also been suggested [12, 41–43].

A study of the biological relevance of AOPP performed in nondialyzed CRF patients showed that AOPP plasma levels were closely related with those of neopterin, a selective monocyte activation marker, and of monocyte-derived pro-inflammatory cytokines, for example, tumor necrosis factor-α and its soluble receptors [3]. In contrast, such a correlation was not found between AOPP and T-cell (CD25) or B-cell (CD23) activation markers. This relationship between AOPPs and monocyte activation was further established by in vitro experiments showing that HSA-AOPP is capable of triggering the respiratory burst of monocytes in normal subjects and that the intensity of the response is proportional to the level of HSA-AOPP oxidation.

Interestingly, we recently observed that in vivo, AOPP levels are closely related with the basal production of ROS by circulating neutrophils and, in vitro, HSA-AOPP is capable of triggering the respiratory burst of isolated neutrophils (abstract; Witko-Sarsat et al, *J Am Soc Nephrol* 8:488A, 1997) [43].

Taken together, these findings allowed us to propose AOPPs as uremic toxins as well as cytokine-like mediators between neutrophils and monocytes. Indeed, as schematically illustrated in Figure 1, neutrophils, which remain the main source of myeloperoxydase involved in the generation of chlorinated oxidants [44], are responsible for the formation of AOPPs, whereas monocytes represent the privileged targets of AOPPs, capable of triggering both respiratory burst and cytokine synthesis and thereby amplify the inflammatory process. Of note, an autoregulatory loop could also occur via the AOPP stimulating effect of neutrophil respiratory burst. An alternative hypothesis is that plasma AOPPs result from

the enzymatic activity of monocyte-derived myeloperoxidase, which might be overactive in uremia.

The molecular mechanisms underlying the biological actions of AOPPs on monocytes remain to be determined. It is highly suggestive that, as previously described for AGEs, AOPP-mediated biological effects involve a ligand–receptor type of interaction, and this is currently under study in our laboratory.

CONCLUSION

The presence of high circulating levels of oxidatively-modified protein products such as AOPP in CRF patients, whether or not they are on dialysis, is a good reflection of the state of oxidative stress often found in dialysis-related pathology. We suggest that this oxidative stress exists, although to a more moderate extent, before the initiation of dialysis therapy. The potential importance of such oxidatively modified proteins as compared with other markers of oxidative stress is that they may behave as authentic mediators of inflammation. As such, AOPPs, through their capacity to activate monocytes and macrophages, could play an important role in the uremia-associated immune system dysregulation and dialysis-related inflammatory complications such as accelerated atherosclerosis (Drüeke et al, p. S-114) and amyloid arthropathy, which greatly alter the patient's quality of life (Fig. 1). From a more fundamental point of view, chlorinated oxidants, which to date have been mainly considered as microbicidal species solely produced by neutrophils, might exert immunomodulating activities via their effects on proteins. This new concept, implying oxidation of protein products at the molecular basis of the deleterious effect of oxidants, is remarkably illustrated by the uremia-associated inflammatory process.

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